METHOD OF CELL GROWTH INHIBITION WITH AGNOPROTEIN

Reference to Government Grant

Field of the Invention

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This invention relates to methods of inhibiting cell growth with polyoma virus agnoproteins, especially with agnoprotein from the human neurotropic polyoma virus JCV.

Background of the Invention

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The polyoma viruses (Polyomaviridae) are DNA viruses which infect a variety of species, including man. There are two polyoma viruses that cause disease in humans; BK virus (isolated from an immunosuppressed kidney 20 transplant patient) and the human neurotropic polyomavirus JCV. Other polyoma viruses include the simian polyoma virus SV40, and the murine and avian polyoma viruses.

JCV is an established etiologic agent of progressive multifocal leukoencephalopathy, a fatal demyelinating disease of humans. Alwine JC 25 (1982), *J. Virol.* 42, 798-803. Recent studies point to the association of JCV with several human cancers, including tumors of neural origin. See, *e.g.*, Del Valle L et al., (2001a) *Cancer Res* 61: 4287-4293; Caldarelli-Stefano R et al., (2000) *Human Pathol.* 31: 394-395; and Khalili K et al. (1999), *Lancet* 353: 1152-1153.

30   As with other polyomaviruses, the genome of JCV is comprised of a double-stranded circular DNA that contains three functional regions. These functional regions are the early and late coding genomes, and the non-coding regulatory sequence. Frisque RJ et al. (1984), *J. Virol.* 51: 458-469. The early genome is responsible for expression of the viral regulatory protein T-antigen. 35   The late genome is expressed after DNA replication and results in the

accumulation of the capsid proteins VP1, VP2, and VP3. In addition, the leader sequences of the late genome transcripts encompass an open reading frame encoding the agnoprotein.

5　　During the 1980's, several laboratories studied polyomavirus agnoprotein. In particular the biological function of agnoprotein in SV40-infected monkey kidney cells was investigated. See, *e.g.*, Alwine JC (1982) *supra* and Khalili K (1988), *Proc. Natl. Acad. Sci. USA* 85: 354-358. These studies show that SV40 agnoprotein is important for the late events of the viral lytic cycle. In more recent studies, JCV agnoprotein was shown to interact with

10　　early T-antigen. See, *e.g.*, Safak M et al. (2001), *J. Virol.* 75: 1476-1486. These studies suggest that T-antigen activity in viral gene transcription and DNA replication may be dictated, at least in part, by the interaction of T-antigen with agnoprotein. Thus, agnoprotein appears to have an integral function in polyoma viral replication.

15　　As mentioned above, JCV infection has been linked to various tumors of central nervous system (CNS) origin, including medullablastoma, glioblastoma, and others. Del Valle L et al., (2001a), *supra*; Khalili K (1999), *supra*. Examination of T-antigen expression in the CNS tumor tissue revealed that not all tumor cells express T-antigen. Evaluation of these tumors for other viral

20　　proteins showed a substantial level of agnoprotein in tumors containing the JCV genome. DeValle L et al. (2002), *J. Nat. Cancer Inst.* 94(4): 267-273.

The importance of agnogene expression in brain tumor cells is unknown. One hypothesis holds that interactions of T-antigen and agnoprotein with each other, and with endogenous cellular proteins, may modulate the growth rate of

25　　tumor cells. Nevertheless, it appears from the studies discussed above that JCV agnoprotein is involved in the development and growth of some CNS neoplasms.

Summary of the Invention

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Surprisingly, it has now been found that polyoma virus agnoproteins inhibit the growth of both normal and abnormally proliferating cells in the absence of other polyoma viral proteins.

The invention thus provides a method of inhibiting cell growth comprising introducing into a cell an effective amount of one or more agnoproteins, or one or more biologically active fragments or derivatives of agnoprotein, such that growth of the cell is inhibited.

5   The invention also provides a method of treating a subject having a cancer or a non-cancerous proliferative disorder, comprising administering to the subject an effective amount of one or more agnoproteins, or one or more biologically active fragments or derivatives of agnoprotein, such that growth of cells of the cancer or non-cancerous proliferative disorder is inhibited.

10   The invention also provides a pharmaceutical composition for treating a subject having a cancer or a non-cancerous proliferative disorder, comprising agnoprotein, or a biologically active fragment or derivative of agnoprotein, and a pharmaceutically acceptable carrier.

The invention further provides a pharmaceutical composition for treating a subject having a cancer or a non-cancerous proliferative disorder, comprising a

15 nucleic acid sequence encoding agnoprotein, or a biologically active fragment or derivative of agnoprotein, and a pharmaceutically acceptable carrier.

Amino Acid Abbreviations

20   The nomenclature used to describe the peptide compounds of the present invention follows the conventional practice wherein the amino group is presented to the left and the carboxy group to the right of each amino acid residue.   In the formulae representing selected specific embodiments of the present invention, the amino-and carboxy-terminal groups, although not

25 specifically shown, will be understood to be in the form they would assume at physiologic pH values, unless otherwise specified.   In the amino acid structure formulae, each residue is generally represented by a one-letter or three-letter designation, corresponding to the trivial name of the amino acid, in accordance with the following schedule:

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A	Alanine	Ala
C	Cysteine	Cys
D	Aspartic Acid	Asp
E	Glutamic Acid	Glu
F	Phenylalanine	Phe
G	Glycine	Gly
H	Histidine	His
I	Isoleucine	Ile
K	Lysine	Lys
L	Leucine	Leu
M	Methionine	Met
N	Asparagine	Asn
P	Proline	Pro
Q	Glutamine	Gln
R	Arginine	Arg
S	Serine	Ser
T	Threonine	Thr
V	Valine	Val
W	Tryptophan	Trp
Y	Tyrosine	Tyr

Definitions

The expression "**amino acid**" as used herein is meant to include both natural and synthetic amino acids, and both D and L amino acids. "**Standard amino acid**" means any of the twenty standard L-amino acids commonly found in naturally occurring peptides. "**Nonstandard amino acid**" means any amino acid, other than the standard amino acids, regardless of whether it is prepared synthetically or derived from a natural source. As used herein, "synthetic amino acid" also encompasses chemically modified amino acids, including but not limited to salts, amino acid derivatives (such as amides), and substitutions. Amino acids contained within the peptides of the present invention, and particularly at the carboxy- or amino-terminus, can be modified by methylation, amidation, acetylation or substitution with other chemical groups which can change the peptide's circulating half life without adversely affecting their biological activity. Additionally, a disulfide linkage can be present or absent in the peptides of the invention.

Amino acids have the following general structure:

$$R \underset{\underset{NH_2}{|}}{\overset{\overset{H}{|}}{C}} COOH$$
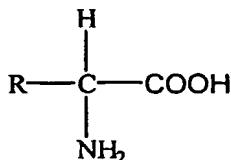
Amino acids are classified into seven groups on the basis of the side chain R: (1) aliphatic side chains, (2) side chains containing a hydroxylic (OH) group, (3) side chains containing sulfur atoms, (4) side chains containing an acidic or amide group, (5) side chains containing a basic group, (6) side chains containing an aromatic ring, and (7) proline, an imino acid in which the side chain is fused to the amino group.

As used herein, **"agnoprotein"** means the approximately 60-75 amino acid protein produced from the gene in the late leader region of a polyoma virus, which is located just upstream of the VP2 structural gene. Agnoproteins useful in the practice of the present invention are "biologically active" as defined herein, and have at least about 50% sequence identity, preferably at least about 60% sequence identity, for example 63% or 64% sequence identity, to SEQ ID NO: 1. More preferably, agnoproteins useful in the invention are biologically active as defined herein and have at least about 80% sequence identity, for example 83% or 84% sequence identity, with SEQ ID NO: 1. Particularly preferred agnoproteins are biologically active as defined herein and have 90%, 95% or 98% sequence identity with SEQ ID NO: 1.

"Biologically active" with respect to agnoprotein, or fragments or derivatives of agnoprotein, means the ability of the compound to inhibit the growth of NIH-3T3 cells according to the *in vitro* cell growth assays given in Example 2 below. Agnoproteins useful in the practice of the present invention specifically include agnoproteins from JCV, BK and SV40 polyoma virus strains.

"Isolated" means altered or removed from the natural state through the actions of a human being. For example, a nucleic acid sequence or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid

or peptide partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated nucleic acid sequence or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

As used herein, **"protecting group"** with respect to a terminal amino group of a peptide means any of the various amino-terminal protecting groups traditionally employed in peptide synthesis. Such protecting groups include, for example, acyl protecting groups such as formyl, acetyl, benzoyl, trifluoroacetyl, succinyl, and methoxysuccinyl; aromatic urethane protecting groups such as benzyloxycarbonyl; and aliphatic urethane protecting groups, for example, tert-butoxycarbonyl or adamantyloxycarbonyl. See Gross and Mienhofer, eds., *The Peptides*, vol. 3, pp. 3-88 (Academic Press, New York, 1981) for suitable protecting groups.

As used herein, **"protecting group"** with respect to a terminal carboxy group of a peptide means any of various carboxyl-terminal protecting groups traditionally employed in peptide synthesis. Such protecting groups include, for example, tert-butyl, benzyl or other acceptable groups linked to the terminal carboxyl group through an ester or ether bond.

"Derivative" includes any naturally occurring or purposefully generated agnoprotein which is characterized by single or multiple amino acid substitutions, deletions, additions, or replacements. Such derivatives include (a) derivatives in which one or more amino acid residues of agnoprotein are substituted with conservative or non-conservative amino acids; (b) derivatives in which one or more amino acids are added; (c) derivatives in which one or more of the amino acids include a substituent group; (d) derivatives in which agnoprotein or a portion thereof is fused to another peptide (e.g., serum albumin or protein transduction domain); (e) derivatives in which one or more nonstandard amino acid residues (*i.e.*, those other than the 20 standard L-amino acids found in naturally occurring proteins) are incorporated or substituted into the agnoprotein sequence; and (f) derivatives in which one or more nonamino acid linking groups are incorporated into or replace a portion of agnoprotein.

"Peptide" and "protein" are used interchangeably, and refer to a compound comprised of at least two amino acid residues covalently linked by peptide bonds or modified peptide bonds (*e.g.*, peptide isosteres). No limitation is placed on the maximum number of amino acids which can comprise a protein or peptide. The amino acids comprising the peptides or proteins described herein and in the appended claims are understood to be either D or L amino acids with L amino acids being preferred. The amino acid comprising the peptides or proteins described herein can also be modified either by natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Modifications can occur anywhere in a peptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It is understood that the same type of modification can be present in the same or varying degrees at several sites in a given peptide. Also, a given peptide can contain many types of modifications. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. See, for instance, PROTEINS--STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter et al., "Analysis for protein modifications and nonprotein cofactors", Meth Enzymol (1990)

182:626-646 and Rattan et al., "Protein Synthesis: Posttranslational Modifications and Aging", Ann NY Acad Sci (1992) 663:48-62.

"Variant" as the term is used herein, is a nucleic acid sequence or peptide that differs from a reference nucleic acid sequence or peptide respectively, but retains essential properties of the reference molecule. Changes in the sequence of a nucleic acid variant can not alter the amino acid sequence of a peptide encoded by the reference nucleic acid, or can result in amino acid substitutions, additions, deletions, fusions and truncations. Changes in the sequence of peptide variants are typically limited or conservative, so that the sequences of the reference peptide and the variant are closely similar overall and, in many regions, identical. A variant and reference peptide can differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A variant of a nucleic acid or peptide can be a naturally occurring such as an allelic variant, or it can be a variant that is not known to occur naturally. Non-naturally occurring variants of nucleic acids and peptides can be made by mutagenesis techniques or by direct synthesis.

As used herein, "sequence identity" with respect to a reference peptide can be computed by using the BLASTP and TBLASTN programs which employ the BLAST (basic local alignment search tool) 2.0.14 algorithm; BLASTP and TBLASTN settings to be used in such computations are indicated in Table 1 below. Amino acid sequence identity is reported under "Identities" by the BLASTP and TBLASTN programs. Techniques for computing amino acid sequence identity are well known to those skilled in the art, and the use of the BLAST algorithm is described in Altschul et al. (1990), *J. Mol. Biol.* 215: 403-10 and Altschul et al. (1997), *Nucleic Acids Res.* 25:3389-3402, the disclosures of which are herein incorporated by reference in their entirety. BLASTP and TBLASTN programs utilizing the BLAST 2.0.14 algorithm and can be accessed at http://www.ncbi.nlm.nih.gov/.

Table 1 - Settings to be used for the computation of amino acid sequence identity with BLASTP and TBLASTN programs utilizing the BLAST 2.0.14 algorithm.

Expect Value	10
Filter	Low complexity filtering using SEG program*
Substitution Matrix	BLOSUM62
Gap existence cost	11
Per residue gap cost	1
Lambda ratio	0.85
Word size	3

*The SEG program is described by Wootton and Federhen (1993), *Comput. Chem.* 17: 149-163.

"**Sequence identity**" with respect to a reference nucleic acid sequence can be determined by comparing the sequence identity of two sequences, for example by physical/chemical methods (*i.e.*, hybridization) or by sequence alignment via computer algorithm. Suitable nucleic acid hybridization conditions to determine if a nucleic acid sequence possesses the required sequence identity to a reference nucleic acid sequence are: 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50 °C with washing in 2X standard saline citrate (SSC), 0.1% SDS at 50 °C; preferably in 7% (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50 °C with washing in 1XSSC, 0.1% SDS at 50 °C. Suitable computer algorithms to determine substantial similarity between two nucleic acid sequences include, but are not limited to: GCS program package (Devereux et al. (1984), *Nucl. Acids Res.* 12: 387), and the BLASTN or FASTA programs (Altschul et al. (1990), *J. Mol. Biol.* 215: 403). The default settings provided with these programs are adequate for determining substantial similarity of nucleic acid sequences for purposes of the present invention.

"**Substantially purified**" refers to a peptide or nucleic acid sequence which is substantially homogenous in character due to the removal of other compounds (*e.g.*, other peptides, nucleic acids, carbohydrates, lipids) or other cells originally present. "Substantially purified" is not meant to exclude artificial or synthetic mixtures with other compounds, or the presence of impurities which do not interfere with biological activity, and which can be present, for example, due to incomplete purification, addition of stabilizers, or formulation into a pharmaceutically acceptable preparation.

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Brief Description of the Figures

FIG. 1A is Western blot analysis of protein extracts from U-87MG cells transfected with a plasmid expressing agnoprotein. Lane 1 represents approximately 50 µg of total protein extract from untransfected cells. Lanes 2-5 represent, respectively, 50 µg of total protein extract from cells transfected with pCMV-agnoprotein at 19, 24, 43, and 49 hours after transfection. Lanes 6 and 7 represent extracts from uninfected and JCV-infected human astrocytes, respectively. The position of the agnoprotein is shown by an arrow. The asterisk shows the position of a non-specific band. **FIGS. 1B** and **1C** are photomicrographs showing U-87MG cells transfected with a plasmid expressing agnoprotein which were fixed and reacted, respectively, with preimmune or anti-agnoprotein antibodies at 24 hrs. post-transfection (original magnification 400X).

FIG. 2A is a plot comparing proliferation of NIH 3T3 cells in the absence and presence of agnoprotein expression. Four independent samples were tested after 24, 48, and 72 hours for cell proliferation by direct counting of the total number of cells. All error is represented in SEM. **FIGS. 2B and 2C** are, respectively, fluorescence-activated cell sorting (FACS) analyses of NIH 3T3 cells stably expressing YFP or YFP-agnoprotein at 0, 16, 21, 25 and 29 hours after serum stimulation. The percentage of cells in G1, S, and G2/M is indicated for each time point. Histograms are representative of three experiments and the average values are presented.

FIGS. 3A and 3B are, respectively, Western blot analyses of cyclin A expression in synchronized NIH 3T3 cells expressing YFP or YFP-agnoprotein at 0, 4, 16, 21 and 29 hours after serum stimulation. 50 µg total protein extract was analyzed for each cell type. As a control for protein loading in the gels, the levels of Grb2 are shown in the bottom panels. **FIG. 3C** is a plot showing associated kinase activity of cyclin A in 220 µg of protein extracted from YFP and YFP-agnoprotein producing NIH 3T3 cells at 0, 4, 16, 21 and 29 hours after serum stimulation. Kinase activity is expressed as counts-per-minute (cpm) on the Y axis, and the time after serum stimulation is given in hours on the X axis.

The kinase assay was repeated two times for each sample, giving an interassay standard deviation within 10% after normalization for protein amount.

FIGS. 4A and 4B are, respectively, Western blot analyses of cyclin B expression in synchronized NIH 3T3 cells expressing YFP or YFP-agnoprotein at 0, 4, 16, 21 and 29 hours after serum stimulation. 50 μg total protein extract was analyzed for each cell type. As a control for protein loading in the gels, the levels of Grb2 are shown in the bottom panels. FIG. 4C is a plot showing associated kinase activity of cyclin B in 220 μg of protein extracted from YFP and YFP-agnoprotein producing NIH 3T3 cells at 0, 4, 16, 21 and 29 hours after serum stimulation. Kinase activity is expressed as counts-per-minute (cpm) on the Y axis, and the time after serum stimulation is given in hours on the X axis. Kinase activity is expressed as the average of two experiments after normalization for protein levels.

FIGS. 5A-C are, respectively, Western blot analyses of approximately 50 μg of total protein extracted from NIH 3T3 ("control") and NIH 3T3-agnoprotein ("Agnoprotein") producing cell lines using antibodies that recognize p27, p21 and β-actin. FIG. 5D is plot of luciferase activity in Saos-2 cells transfected with a p21-luciferase reporter gene alone, or with plasmids expressing p53 ("p53 +/-") or agnoprotein ("Agnoprotein +/-").

FIG. 6A is a Western blot analysis of 50 μg protein extracted from control and agnoprotein-producing NIH 3T3 cells using anti-p53 antibody FIG. 6B is an autoradiograph of agnoprotein that was immobilized on glutathione-Sepharose beads, incubated with *in vitro* translated [^{35}S]-methionine-labeled p53 and resolved by SDS-PAGE. One-tenth of the input p53 used in each reaction was loaded as a migrating control in lane 1. FIG. 6C is an autoradiograph of p53 fused to GST beads mixed with *in vitro* translated [^{35}S]-labeled agnoprotein and resolved by SDS-PAGE. One-tenth of the of the input from the *in vitro* translation reaction was loaded as a migrating control in lane 1. FIG. 6D is a Western blot analysis of immunoprecipitates from 150 μg protein extracted from control NIH 3T3 and agnoprotein-producing NIH 3T3 cells. The position of the fusion agnoprotein is shown by an arrow. The asterisk indicates a non-specific band seen in both control and experimental cell extracts. FIG. 6E is a

Western blot analysis of immunoprecipitates from approximately 250 µg protein extracted from control cells expressing YFP or cells expressing GFP-Agnoprotein were analyzed by GST pull-down assay using GST or GST-p53 as indicated. Lanes 1 and 2 represent an analysis of 50 µg protein extract from each cell type which was used in the binding experiment. The arrow indicates the position of YFP-Agnoprotein. **FIG. 6F** is an autoradiograph of *in vitro* [^{35}S]-methionine-labeled p53 protein analyzed by GST pull-down assay using various regions of the agnoprotein as indicated, which were fused to GST. The position of the labeled p53 is indicated by the arrow.

Detailed Description of the Invention

Introduction of agnoprotein into a cell, in the absence of other polyoma viral proteins, inhibits growth of the cell. Without wishing to be bound by any particular theory, it appears that agnoprotein deregulates cell growth by delaying progression of cells through the various phases of the cell cycle. The growth of both normally and abnormally proliferating cells can be inhibited with agnoprotein.

Agnoprotein from any species and strain of polyoma virus can be used in the practice of the present invention. Preferred agnoproteins are those derived from JCV, in particular from the Mad-1 strain of JCV. The agnoprotein from JCV strain Mad1 is a 71 amino acid protein with the following primary sequence:

MVLRQLSRKASVKVSKTWSGTKKRAQRILIFLLEFLLDFCTGEDS VDGKKRQRHSGLTEQTYSALPEPKAT (SEQ ID NO: 1)

The nucleic acid sequence encoding SEQ ID NO: 1 is given in SEQ ID NO: 2.

Other JCV agnoproteins known in the art include those disclosed in GenBank records Accession No. AF187236 (SEQ ID NO: 3), Accession No. AF281599 (SEQ ID NO: 4), Accession No. AF187234 (SEQ ID NO: 5), Accession No. AF295737 (SEQ ID NO: 6) and Accession No. AF295739 (SEQ

ID NO: 7), the entire disclosures of which are herein in incorporated by reference. The nucleic acid sequences encoding these agnoproteins are given, respectively, in SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12.

5   See also Cubitt CL et al. (2001), *J. Neurovirol.* *7*: 339-344, the disclosure of which is herein incorporated by reference, in which predicted agnoprotein amino acid sequences for 100 JCV strains were analyzed to produce the following agnoprotein consensus sequence:

M-V-L-R-Q-L-S-R-K-A-S-V-K-V-S-K-T-W-S-G-T-K-K-R-A-Q-R-I-L-
10   I-F-L-L-E-F-L-L-D-F-C-T-G-E-D-X_1-V-D-G-K-K-R-Q-X_2-H-X_3-X_4-X_5-X_6-X_7-
X_8-X_9-X_{10}-X_{11}-A-L-P-E-P-K-A-X_{12} (SEQ ID NO: 13)

wherein X_1 is serine or arginine;
                 X_2 is lysine or arginine;
15   X_3 is serine or arginine;
                 X_4 is glycine or no amino acid;
                 X_5 is leucine or no amino acid;
                 X_6 is threonine or no amino acid;
                 X_7 is glutamine, glutamic acid, or no amino acid;
20   X_8 is glutamine or no amino acid;
                 X_9 is threonine, arginine, lysine or no amino acid;
                 X_{10} is tyrosine or no amino acid;
                 X_{11} is serine or glycine; and
                 X_{12} is threonine or lysine.
25
A protein comprising SEQ ID NO: 13 is considered an agnoprotein, and can be used to inhibit growth of cells according to the present invention.

Agnoprotein from human BK polyoma virus strains, or from SV40 polyoma virus strains, are also known. Examples of BK virus agnoproteins are
30   found in GenBank record Accession Nos. M23122 (SEQ ID NO: 14) and D00678 (SEQ ID NO: 15), and a partial BK virus agnoprotein sequence is found in Accession No. AF442903 (SEQ ID NO: 16), the disclosures of which are herein incorporated by reference. An example of SV40 agnoprotein is found in GenBank record Accession No. M99359 (SEQ ID NO: 17), the disclosure of
35   which is herein incorporated by reference.

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Nucleic acid sequences encoding the BK virus and SV40 agnoproteins described above are given in SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, and SEQ ID NO: 21 respectively.

Agnoproteins can be isolated from mammalian cells infected with polyoma viruses according to known techniques. Agnoproteins can also be produced synthetically by any known means, including synthesis by biological systems and by chemical methods.

Biological synthesis of peptides is well known in the art, and includes the transcription and translation of a naturally-occurring or synthetic gene encoding agnoprotein sequences. These nucleic acids can be subcloned into an appropriate plasmid expression vector for propagation and expression in an appropriate host. For example, techniques for cloning and expression of the agnoprotein of SEQ ID NO: 1 are described in the Examples below.

Other techniques used to construct nucleic acid sequences and plasmid expression vectors, transfect host cells, and express a nucleic acid sequence of interest are widely practiced in the art, and practitioners of ordinary skill are familiar with the standard resource materials which describe specific conditions and procedures. For example, general methods for the cloning and expression of recombinant molecules are described in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratories, 1982; and in Ausubel, Current Protocols in Molecular Biology, Wiley and Sons, 1987, the disclosures of which are incorporated herein by reference.

Agnoprotein produced from an expression vector can be obtained from the host cell by cell lysis, or by using heterologous signal sequences fused to the expressed protein which cause secretion of the protein into the surrounding medium. Preferably, the signal sequence is designed so that it can be removed by chemical or enzymatic cleavage, as is known in the art. The agnoprotein thus produced can then be purified in a manner similar to that utilized for isolation of agnoprotein from isolated from mammalian cells infected with polyoma viruses.

Chemical peptide synthesis techniques suitable for directly synthesizing agnoprotein, including manual and automated techniques, are also well-known to those of ordinary skill in the art. For example, agnoprotein can be

synthesized *de novo* using conventional solid phase synthesis methods. In such methods, the peptide chain is prepared by a series of coupling reactions in which the constituent amino acids are added to the growing peptide chain in the desired sequence. The use of various N-protecting groups, *e.g.*, the carbobenzyloxy group or the *t*-butyloxycarbonyl group; various coupling reagents *e.g.*, dicyclohexylcarbodiimide or carbonyldimidazole; various active esters, *e.g.*, esters of N-hydroxyphthalimide or N-hydroxy-succinimide; and the various cleavage reagents, *e.g.*, trifluoroactetic acid (TFA), HCl in dioxane, boron tris-(trifluoracetate) and cyanogen bromide; and reaction in solution with isolation and purification of intermediates are methods well-known to those of ordinary skill in the art.

A preferred chemical peptide synthesis method follows conventional Merrifield solid phase procedures well known to those skilled in the art. Additional information about solid phase synthesis procedures can be had by reference to Steward and Young, Solid Phase Peptide Synthesis, W.H. Freeman & Co., San Francisco, 1969; the review chapter by Merrifield in Advances in Enzymology 32:221-296, (Nold FF, ed.), Interscience Publishers, New York, 1969; and Erickson and Merrifield (1990), *The Proteins* 2:61-64, the entire disclosures of which are incorporated herein by reference. Crude peptide preparations resulting from solid phase syntheses can be purified by methods well known in the art, such as preparative HPLC. The amino-terminus can be protected according to the methods described for example by Yang *et al.*, *FEBS Lett.* 272:61-64 (1990), the entire disclosure of which is herein incorporated by reference.

Agnoproteins for use in the present invention can also comprise a label a (*e.g.*, substances which are magnetic resonance active; radiodense; fluorescent; radioactive; detectable by ultrasound; detectable by visible, infrared or ultraviolet light) so that the agnoprotein can be detected. Suitable labels include, for example, fluorescein isothiocyanate (FITC); peptide chromophores such as phycoerythrin or phycocyanin and the like; bioluminescent peptides such as the luciferases originating from *Photinus pyrali*; fluorescent proteins originating from *Renilla reniformi*; and radionuclides such as ^{32}P, ^{33}P, ^{35}S, I^{125}

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or ^{123}I. For example, the label can comprise an NH_2-terminal fluorescein isothiocyanate (FITC)-Gly-Gly-Gly-Gly motif that is conjugated to a protein transduction domain.

Methods of modifying peptide sequences such as agnoprotein with labels are well known to those skilled in the art. For example, methods of conjugating fluorescent compounds such as fluorescein isothiocyanate to short peptides are described in Danen *et al., Exp. Cell Res.*, 238:188-86 (1998), the entire disclosure of which is incorporated herein by reference.

Biologically active fragments of agnoprotein can also be used in the present methods. Biologically active agnoprotein fragments according to the invention can be obtained, for example, by chemical or enzymatic fragmentation of larger natural or synthetic agnoproteins, or by biological or chemical syntheses as described above.

Biologically active derivatives of agnoprotein can also be used in the present methods. The techniques for obtaining such derivatives are known to persons having ordinary skill in the art and include, for example, standard recombinant nucleic acid techniques, solid phase peptide synthesis techniques and chemical synthetic techniques as described above. Linking groups can also be used to join or replace portions of agnoprotein and other peptides. Linking groups include, for example, cyclic compounds capable of connecting an amino-terminal portion and a carboxyl terminal portion of agnoprotein. Techniques for generating derivatives are also described in U.S. patent 6,030,942 the entire disclosure of which is herein incorporated by reference (derivatives are designated "peptoids" in the 6,030,942 patent). Agnoprotein derivatives can also comprise labels such as are described above.

Agnoprotein derivatives also include fusion peptides in which a portion of the fusion peptide has a substantially similar amino acid sequence to agnoprotein. Such fusion peptides can be generated by techniques well-known in the art, for example by subcloning nucleic acid sequences encoding an agnoprotein and a heterologous peptide sequence into the same expression vector, such that the agnoprotein and the heterologous sequence are expressed together in the same protein. The heterologous sequence can comprise a peptide

leader sequence that directs entry of the expressed protein into a cell. Such leader sequences include "protein transduction domains" or "PTDs", which are discussed in more detail below.

A preferred agnoprotein derivative comprises the JCV agnoprotein described in Jobes DV et al. (1999), *J. Human Virol.* 2(6): 350-358, the disclosure of which is herein incorporated by reference, which has a 7-amino acid deletion in the C-terminal region (SEQ ID NO: 22). The nucleic acid sequence encoding SEQ ID NO: 22 is given in SEQ ID NO: 23.

The agnoproteins and biologically active fragments and derivatives of agnoproteins described above are also referred to hereunder as "compounds of the invention."

The compounds of the invention can be modified to enhance their entry into cells. For example, the compounds of the invention can be encapsulated in a liposome prior to being administered. The encapsulated compounds are delivered directly into the abnormally proliferating cells by fusion of the liposome to the cell membrane. Reagents and techniques for encapsulating the present compounds in liposomes are well-known in the art, and include, for example, the ProVectin™ Protein Delivery Reagent from Imgenex.

In a preferred embodiment, the compounds of the invention are modified by associating the compounds with a peptide leader sequence known as a "protein transduction domain" or "PTD." These sequences direct entry of the compound into abnormally proliferating cells by a process known as "protein transduction." *See* Schwarze et al. (1999), *Science* 285: 1569 – 1572.

PTDs are well-known in the art, and can comprise any of the known PTD sequences including, for example, arginine-rich sequences such as a peptide of nine to eleven arginine residues optionally in combination with one to two lysines or glutamines as described in Guis et al. (1999), *Cancer Res.* 59: 2577-2580, the disclosure of which is herein incorporated by reference. Preferred are sequences of eleven arginine residues or the NH_2-terminal 11-amino acid protein transduction domain from the human immunodeficiency virus TAT protein (SEQ ID NO: 24). Other suitable leader sequences include, but are not limited to, other arginine-rich sequences; *e.g.*, 9 to 10 arginines, or

six or more arginines in combination with one or more lysines or glutamines. Such leader sequences are known in the art; see, *e.g.*, Guis et al. (1999), *supra.* Preferably, the PTD is designed so that it is cleaved from the compound upon entry into the cell. A PTD can be located anywhere on the agnoprotein, or fragment or derivative of agnoprotein, that does not disrupt the compound's biological activity, and is preferably located at the N-terminal end.

Kits and methods for constructing fusion proteins comprising a protein of interest (*e.g.*, agnoprotein) and a PTD are known in the art; for example the TransVector™ system (Q-BIOgene), which employs a 16 amino acid peptide called "Penetratin™" corresponding to the *Drosophila* antennapedia DNA-binding domain; and the Voyager system (Invitrogen Life Technologies), which uses the 38 kDa VP22 protein from Herpes Simplex Virus-1.

Agnoprotein, or biologically active fragments or derivatives of agnoprotein, can inhibit proliferation of normal and abnormally proliferating cells. Abnormally proliferating cells include cells from cancer types of diverse histologic subtype and origin, such as those listed and described in the National Cancer Institute's "CancerNet" at:

> http://cancernet.nci.nih.gov/pdq/pdq_treatment.shtml

> which is herein incorporated by reference in its entirety.

For example, the compounds of the invention can be used to inhibit the proliferation of primary or metastatic tumor or neoplastic cells from cancers of at least the following histologic subtypes: sarcoma (cancers of the connective and other tissue of mesodermal origin); melanoma (cancers deriving from pigmented melanocytes); carcinoma (cancers of epithelial origin); adenocarcinoma (cancers of glandular epithelial origin); cancers of neural origin (glioma/glioblastoma and astrocytoma); and hematological neoplasias, such as leukemias and lymphomas (*e.g.*, acute lymphoblastic leukemia, chronic lymphocytic leukemia, and chronic myelocytic leukemia).

The compounds of the invention can also be used to inhibit the proliferation of primary or metastatic tumor or neoplastic cells from cancers having their origin in at least the following organs or tissues, regardless of histologic subtype: breast; tissues of the male and female urogenital system (*e.g.*

ureter, bladder, prostate, testis, ovary, cervix, uterus, vagina); lung; tissues of the gastrointestinal system (*e.g.*, stomach, large and small intestine, colon, rectum); exocrine glands such as the pancreas and adrenals; tissues of the mouth and esophagus; brain and spinal cord; kidney (renal); pancreas; hepatobiliary system (*e.g.*, liver, gall bladder); lymphatic system; smooth and striated muscle; bone and bone marrow; skin; and tissues of the eye.

Furthermore, the compounds of the invention can be used to inhibit the proliferation of cells from cancers or tumors in any prognostic stage of development, as measured, for example, by the "Overall Stage Groupings" (also called "Roman Numeral") or the Tumor, Nodes, and Metastases (TNM) staging systems. Appropriate prognostic staging systems and stage descriptions for a given cancer are known in the art, for example as described in:

http://cancernet.nci.nih.gov/pdq/pdq_treatment.shtml, *supra*.

Agnoprotein, or biologically active fragments or derivatives of agnoprotein, can also be used to inhibit proliferation of cells from non-cancerous proliferative disorders. The non-cancerous proliferative disorders are characterized by cells which have escaped normal growth controls, but are not able to metastasize. Abnormally proliferating cells in such disorders typically form fibroid growths or benign tumors.

Examples of non-cancerous proliferative disorders include any benign skin lesion or condition involving the uncontrolled growth of fibroblasts, hemangiomatosis in newborn; secondary progressive multiple sclerosis; chronic progressive myelodegenerative disease; neurofibromatosis; ganglioneuromatosis; keloid formation; Paget's Disease of the bone; fibrocystic disease (*e.g.*, of the breast, lungs, or uterus); sarcoidosis; Peronies' and Duputren's fibrosis, cirrhosis, atherosclerosis and vascular restenosis.

Preferably, the compounds of the invention are used to treat a subject having abnormally proliferating cells deriving from a cancer or a non-cancerous proliferative disorder. In treating subjects with such conditions, one or more compounds of the invention are administered to a subject in an amount effective to inhibit proliferation of abnormally proliferating cells (the "effective

amount"). The subject can be any animal, preferably a mammal, particularly preferably a human being.

As used herein, to "inhibit the proliferation of an abnormally proliferating cell" means to kill the cell, or permanently or temporarily arrest the growth of the cell.

Inhibition of proliferation can be inferred if the number of abnormally proliferating cells in the subject remains constant or decreases after administration of the present compounds. Inhibition of proliferation can also be inferred if the absolute number of abnormally proliferating cells increases, but the rate of growth of a tissue mass decreases. As used herein, a "tissue mass" is any localized collection of abnormally proliferating cells in a subject's body; for example a tumor, fibroid body, restenotic plaque, and the like. The number of abnormally proliferating cells in a subject's body can be determined by direct measurement (e.g., calculating the concentration of leukemic cells in the blood or bone marrow) or by estimation from the size of a tissue mass.

The size of a tissue mass can be ascertained by direct visual observation or by diagnostic imaging methods such as X-ray, magnetic resonance imaging, ultrasound, and scintigriphy. Diagnostic imaging methods used to ascertain size of a tissue mass can be employed with or without contrast agents, as is known in the art. The size of a tissue mass can also be ascertained by physical means, such as palpation of the tissue mass or measurement of the tissue mass with a measuring instrument such as a caliper.

Agnoprotein, or biologically active fragments or derivatives of agnoprotein, can be administered to a subject by any technique designed to expose abnormally proliferating cells in the subject's body to the compounds, such that the compounds are taken up by the cells. For example, the compounds of the invention can be administered by any enteral or parenteral route. Parenteral administration is preferred.

Suitable parenteral administration methods include intravascular administration (e.g. intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature); peri- and intra-tissue injection (e.g. peri-tumoral and intra-tumoral

injection); subcutaneous injection or deposition including subcutaneous infusion (such as by osmotic pumps); and direct application to the abnormally proliferating cells or to tissue comprising the abnormally proliferating cells, for example by a catheter or other placement device. It is preferred that subcutaneous injections or infusions be given in the area near the abnormally proliferating cells, particularly if the cells are on or near the skin.

The compounds of the invention can be injected in a single dose or in multiple doses. Infusion of the compounds of the invention can comprise a single sustained dose over a prolonged period of time or multiple infusions. Direct injection into tissue comprising the abnormally proliferating cells is preferred.

An effective amount of the compounds of the invention can be based on the approximate weight of the tissue mass to be treated. The approximate weight of a tissue mass can be determined by calculating the approximate volume of the tissue mass, wherein one cubic centimeter of tissue mass volume is roughly equivalent to one gram. Where more than one compound of the invention is administered, the effective amount represents the cumulative total of the administered compounds.

An effective amount of the compounds of the invention based on the weight of a tissue mass can be at least about 10 μg compound/gram of tissue mass, and is preferably between about 10-1000 μg compound/gram of tissue mass. More preferably, the effective amount is at least about 60 μg compound/gram of tissue mass. Particularly preferably, the effective amount is at least about 100 μg compound/gram of tissue mass. It is preferred that effective amounts based on the weight of the tissue mass be injected directly into the tissue mass.

An effective amount of the compounds of the invention can also be based on the approximate or estimated body weight of the subject to be treated. Preferably, such effective amounts are administered systemically; e.g. by intravascular injections and infusions, subcutaneous depositions or infusions, or intramuscular or intraperitoneal administrations. Where more than one

compound of the invention is administered, the effective amount represents the cumulative total of the administered compounds.

For example, an effective amount of the compounds of the invention administered by single intravascular injection in humans (assuming a 60 kg subject) can range from about 5 – 3000 µg compound/kg of body weight, is preferably between about 700 - 1000 µg compound/kg of body weight, and is more preferably greater than about 1000 µg compound/kg of body weight.

An effective amount of the compounds of the invention used for multiple intravascular injections can be the same or lower than that used for single intravascular injections. For example, an effective amount for multiple intravascular injection in humans is preferably greater than about 250 µg compound/kg body weight, and is more preferably greater than about 500 µg compound/kg body weight.

An effective amount of the compounds of the invention administered by single sustained intravascular infusion can be the same as that used for single and multiple intravascular injections, but can also be lower. For example, an effective amount for single sustained infusions in humans is preferably greater than about 90 µg compound/kg body weight, and is more preferably greater than about 100 µg compound/kg body weight.

An effective amount of the compounds of the invention administered by multiple sustained intravascular infusions can be the same as that used for single and multiple injections and single sustained intravascular infusion, but can also be lower. For example, an effective amount for multiple sustained intravascular infusions in humans is preferably greater than about 35 µg/kg, and is more preferably greater than about 50 µg/kg.

An effective amount of the compounds of the invention administered by subcutaneous, intramuscular or intraperitoneal routes can be the same as that used for intravascular administration, but is preferably between about 200 and 1000 µg compound/kg body weight. More preferably, the effective amount is greater than 500 µg compound/kg of body weight.

An effective amount of the compounds of the invention can also be based on the approximate surface area of the subject to be treated. Effective amounts based on surface area are typically expressed in terms of µg compound/square meter of surface area (m^2). It is preferred to base effective

5   amounts on the surface area of a subject, because better inter-species comparisons can be made. Also, effective amounts based on surface area allow amounts to be determined for human adults and children without further adjustment. The assumptions underlying the inter-species and adult to child conversion of effective amounts based on surface area are found in EJ Freireich

10   *et al.*, (1966), *Cancer Chemotherapy Reports* 50: 219-244, the disclosure of which is herein incorporated by reference in its entirety.

Table 2 provides approximate surface area-to-weight ratios for various species. The surface area-to-weight ratio can be used to convert effective amounts based on body weight (expressed in µg/kg) to effective amounts based

15   on surface area (expressed in µg/m^2). The surface area-to-weight ratio is also used to calculate the conversion factors found in Table 3, which can be used to convert effective amounts expressed in terms of µg/kg from one species to another.

20   Table 2 - Surface Area to Weight Ratios of Various Species[*]

SpeciesBody	Weight (kg)	Surface Area (m^2)	Surface Area to Weight Ratio (kg/m^2)
Mouse	0.02	0.0066	3.0
Rat	0.15	0.025	5.9
Monkey	3	0.24	12
Dog	8	0.40	20
Human			
child	20	0.80	25
adult	60	1.6	37

[*]Adapted from DeVita, VT, "Principles of Chemotherapy," pgs. 292-3, in Cancer: Principles and Practice of Oncology, (3rd edit., DeVita VT, Hellman S, and Rosenberg SA, eds.), 1989, J. B. Lipincott Co., Phila., PA.

As shown in Table 2, to convert an effective amount based on body weight in µg/kg for any given species to the equivalent effective amount based

on surface area (in $\mu g/m^2$), multiply the effective amount based on body weight by the approximate surface area to weight ratio. For example, in the adult human 100 $\mu g/kg$ is equivalent to 100 $\mu g/kg \times 37$ kg/m^2 = 3700 $\mu g/m^2$.

Table 3 gives approximate factors for converting effective amounts expressed in terms of $\mu g/kg$ from one species to an equivalent *surface area* effective amount expressed in the same units ($\mu g/kg$) for another species. For example, given a dose of 50 $\mu g/kg$ in the mouse, the appropriate dose in man (assuming equivalency on the basis of $\mu g/m^2$) is 50 $\mu g/kg \times 1/12 = 4.1$ $\mu g/kg$. For the present invention, equivalency on the basis of $\mu g/m^2$ is assumed.

Table 3 - Equivalent Surface Area Dosage Conversion Factors[*]

	Mouse (20g)	Rat (150g)	Monkey (3 kg)	Dog (8 kg)	Man (60 kg)
Mouse	1	1/2	1/4	1/6	1/12
Rat	2	1	1/2	1/4	1/7
Monkey	4	2	1	3/5	1/3
Dog	6	4	5/3	1	1/2
Man	12	7	3	2	1

[*]Adapted from DeVita, VT, "Principles of Chemotherapy," pgs. 292-3, in Cancer: Principles and Practice of Oncology, (3rd edit., DeVita VT, Hellman S, and Rosenberg SA, eds.), 1989, J. B. Lipincott Co., Phila., PA.

An effective amount of the compounds of the invention based on surface area is preferably administered systemically, as described above for effective amounts based on body weight. However, effective amounts based surface area can also be administered by peri- or intra-tissue mass injection or by direct application to the tissue mass. Where more than one compound of the invention is administered, the effective amount represents the cumulative total of the administered compounds.

Agnoprotein, and biologically active fragments and derivatives of agnoprotein, can also be administered to a subject by transfection of the abnormally proliferating cells in the subject's body with one or more nucleic acid sequences encoding a compound of the invention. Preferably, the nucleic acid sequences comprise a plasmid expression vector. Such plasmids can be

generated by recombinant nucleic acid and molecular cloning techniques well-known in the art, as discussed above.

Transfection methods for eukaryotic cells are well known in the art, and include, for example, direct injection of the nucleic acid into the nucleus or
5   pronucleus; electroporation; liposome transfer; receptor mediated nucleic acid delivery, bioballistic or particle acceleration; calcium phosphate precipitation, and transfection mediated by viral vectors.

For example, the transfection is performed with a liposomal transfer compound, e.g., DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-
10   ammonium methylsulfate, Boehringer - Mannheim) or an equivalent, such as LIPOFECTIN. The amount of nucleic acid used is not critical to the practice of the invention; acceptable results can be achieved with 10 mM nucleic acid/10^5 cells. A ratio of about 500 nanograms of plasmid vector in 3 micrograms of DOTAP per 10^5 cells can be used. Other suitable methods for the construction
15   and propagation of plasmid vectors capable of expressing the present compounds, and techniques for transfecting such vectors into eukaryotic cells so that the compounds are expressed, are known in the art. A preferred transfection method is the calcium phosphate precipitation method described in the Examples below.

20   The present invention also provides pharmaceutical formulations for treating cancer or non-cancerous proliferative disorders. The pharmaceutical compositions of the invention comprise one or more agnoproteins, or one or more biologically active fragments or derivatives of agnoprotein. The pharmaceutical formulations of the present invention can also comprise one or
25   more nucleic acids encoding an agnoprotein or a biologically active fragment or derivative of agnoprotein.

Pharmaceutical formulations of the present invention are characterized as being at least sterile and pyrogen-free. As used herein, "pharmaceutical formulations" include formulations for human and veterinary use. The present
30   pharmaceutical formulations can be prepared by mixing agnoprotein, biologically active fragments or derivatives of agnoprotein, or nucleic acids encoding these compounds with a physiologically acceptable carrier medium to

form solutions, suspensions or dispersions. Preferred physiologically acceptable carrier media are water or normal saline.

Pharmaceutical formulations of the invention can also comprise conventional pharmaceutical excipients and/or additives. Suitable pharmaceutical excipients include stabilizers, antioxidants, osmolality adjusting agents, buffers, and pH adjusting agents. Suitable additives include physiologically biocompatible buffers (e.g., tromethamine hydrochloride), additions (e.g., 0.01 to 10 mole percent) of chelants (such as, for example, DTPA or DTPA-bisamide) or calcium chelate complexes (as for example calcium DTPA, CaNaDTPA-bisamide), or, optionally, additions (e.g. 1 to 50 mole percent) of calcium or sodium salts (for example, calcium chloride, calcium ascorbate, calcium gluconate or calcium lactate). Pharmaceutical formulations of the invention can be prepared in a manner fully within the skill of the art.

The invention will now be illustrated with the following non-limiting examples.

Examples

General Experimental Procedures

The following general experimental procedures were used in the Examples discussed below.

Cell Culture and Transfection - Human astrocytoma cell line U-87MG (ATCC catalog no. HTB-14), human glioblastoma multiforme cell line T98G (ATCC catalog no. CRL-1960), mouse fibroblast NIH 3T3 cells (ATCC catalog no. CRL-1658), and cell line Saos-2 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections were performed on approximately 5-7 x 10^5 cells per 60 mm dish using the calcium phosphate precipitation method, according to the method of Graham FL and van der Eb AJ (1973) *Virology* 52, 456-467, the entire disclosure of which is herein incorporated by reference. At 48 hours post-transfection, cells were harvested for protein extract preparation or were

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transferred to 100 mm plates containing DMEM plus 10% FBS and Geneticin at a concentration of 0.5 mg/ml for the selection of stably transfected cells.

Plasmids - The plasmids that were used in the various transfections were pCMV-agnoprotein, pCMV-GFP, pGEX-1λT-agnoprotein, pYFP-agnoprotein,
5   pEYFP-N1, pTR(AAV)-Agno, and p21-luciferase.

pCMV-agnoprotein and pGEX-1λT-agnoprotein are described in Safak M et al. (2001) *J. Virol.* **75**, 1476-1486, the entire disclosure of which is herein incorporated by reference.

pEGFP-N1 was obtained from Clontech.
10   pYFP-agnoprotein was created by PCR amplification of the gene encoding the agnoprotein from the plasmid pBJC (which contained the Mad-1 strain of JCV) using the following primers containing a BamHI and EcoRI site, respectively:

15   5'-ACGTCCAGGATCCATGGTTCTTCGCCAGCTGTCA-3'
         (SEQ ID NO: 25)
         5'-ACGTCCAGAATTCCTATGTAGCTTTTGGTTCAGG-3'
         (SEQ ID NO: 26)

20   After gel purification, the amplicon was digested with BamHI and EcoRI and subcloned into the same sites of the pEYFP-N1 vector multiple cloning site (Clontech). All constructs were verified by sequencing. The pEYFP-N1 vector, which expresses only YFP, was used as a control.

pTR(AAV)-Agno was constructed by amplifying the sequence encoding
25   the agnoprotein with the following primers, which contain NotI sites and create a T7 promoter for the amplicon:

         5'-TATGCGGCCGCTAATACGACTCACTATAGG-3' (SEQ ID NO: 27)
         5'-TAGAATAGGGCCCTCTAGATGCATGCTCGA-3' (SEQ ID NO: 28)

30

The amplicon was then digested with NotI and inserted into the NotI site of the pTR(AAV) vector. All constructs were verified by sequencing.

p21-luciferase was obtained from Dr. B. Sawaya, Center for Neurovirology and Cancer Biology, Temple University, Philadelphia, PA.

Protein Extract Preparation - For protein extraction, cells were lysed for 20 min on ice in lysis buffer (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.5% NP40) containing 10 µg/ml leupeptin, 2 µg/ml aprotinin, 100 µg/ml PMSF, 100 µg/ml TPCK; and 10 µg/ml pepstatin. Cell debris was removed by centrifugation and the supernatant was collected for protein concentration by Bradford analysis (Bio-Rad, Hercules, CA).

Western Blot Analysis - Approximately 50 µg of the protein extracts were separated by SDS-PAGE (12% gel) and analyzed by Western blot using the specific antibodies set forth in the Examples below. Briefly, proteins resolved by SDS-PAGE were transferred to Optitran-supported nitrocellulose (Schleicher & Schuell) and blocked in PBS-T (PBS-0.1% Tween 20) containing 10% non-fat dry milk for 45 min. Blots were then incubated in primary antibody for two hours, washed 3 x 10 min in PBS-T, incubated with anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase for an additional hour, followed by washing 3 x 10 min in PBS-T. Blots were developed with ECL-Plus (Amersham Pharmacia, Piscataway, NJ) and detected by autoradiography.

Immunoprecipitation - 150 - 500 µg of protein extracts were incubated with anti-p53 antibody overnight at 4° C, with rotation. This solution was then incubated with 30 µl of pre-washed protein A Sepharose for 2 hours at 4° C to precipitate the protein complexes. The precipitated Sepharose A/protein complexes were washed twice in lysis buffer, resuspended in 10 µl of Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8; 2% SDS; 25% glycerol; 0.01% Bromophenol Blue) (Biorad), and incubated for 15 min at room temperature. Samples were centrifuged, the supernatant was boiled at 95° C for 10 min and resolved by SDS-PAGE followed by Western blot analysis as above, using anti-agnoprotein antibody.

H1 kinase assay - Approximately 200 µg of protein extract was immunoprecipitated with antibodies as specified in the Examples below. Approximately 30 µl of packed protein-A Sepharose beads in lysis buffer was

added to the extracts and incubated at 4° C for 3 hours. The immunoprecipitates were assayed for kinase activity for 30 min at 30° C, in assay buffer containing 5 μCi of [γ^{32}P]ATP, 5 μg of histone H1, 1 mM DTT, 10 mM MgCl$_2$, and 20 mM Tris (pH 7.4). The reaction was stopped by the addition of Laemmli sample buffer (BioRad) and analyzed by SDS-PAGE followed by autoradiography.

Cell Proliferation and Flow Cytometry - Cells were plated in 35 mm petri dishes at a density of 1 x 10^4 cells/plate and maintained in DMEM plus 10% FBS. Cells were collected at various times and cell proliferation was assessed by determining total cell number by phase contrast microscopy.

For flow cytometry, cells were synchronized by serum starvation for 72 hrs. and then stimulated by the addition of growth media containing serum. At various times after serum stimulation, as indicated in the Examples, cells were collected by centrifugation and were resuspended in 300 μl of PBS containing 10 μg/ml of propidium iodide and 250 μg/ml of RNase A. Samples were incubated at 37° C for 30 min and kept at 4° C until analyzed. Cells were analyzed by flow cytometry (*i.e.*, fluorescence activated cell sorting, or "FACS") with a Becton Dickinson "FACScan" flow cytometer using the SOBR program.

In Vitro GST Pull-Down Assay - Three microliters of [^{35}S]-labeled *in vitro* translated p53 or agnoprotein were incubated with 5 μg of GST or fusion proteins, GST-agnoprotein or GST-p53 coupled to glutathione Sepharose beads in 300 μl of LB150 buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 0.1% NP40) for one hour at 4° C, with continuous rocking. After incubation, the beads were precipitated and washed five times with LB150 buffer. The bound proteins were eluted with Laemmli sample buffer (BioRad), heated at 95° C for 10 min, and resolved by SDS-PAGE. p53 or agnoprotein was detected by autoradiography.

Example 1 - Expression of Agnoprotein in Glial Cells and Effect of Agnoprotein on Cell Cycle Progression

U-87MG cells were transfected with the agnoprotein-expressing plasmid pCMV-agnoprotein, and the level of agnoprotein was determined at 19, 24, 43

5   and 49 hrs. post-transfection. Untransfected U-87MG cells were used as a control. As shown in Fig. 1A, Western blot analysis of protein extracts from control cells (lane 1) and transfected cells at the various time points (lanes 2-5) showed detectable levels of agnoprotein in transfected cells from 19 hours post-transfection, with accumulation of agnoprotein up to 49 hours post-transfection.

10 Figure 1A also shows a band corresponding to agnoprotein in normal human astrocytes infected with JCV at 15 days post-infection (lane 7), and its absence in uninfected cells (lane 6).

Subcellular localization of agnoprotein in U-87MG cells transfected with pCMV-agnoprotein was also determined. Immunohistochemical examination of

15 transfected U-87MG cells revealed that incubation with pre-immune sera did not detect agnoprotein (Fig. 1B). However, incubation of transfected U-87MG cells with rabbit anti-Agno protein (prepared as described in DeValle L et al. (2002), *J. Nat. Cancer Inst.* 94(4): 267-273, the entire disclosure of which is herein incorporated by reference) showed cytoplasmic perinuclear accumulation of

20 agnoprotein in the cells (Fig. 1C).

To determine the effect of agnoprotein expression on cell cycle progression, U-87MG cells were synchronized and serum stimulated as above. At 4 hrs. after serum stimulation, cells were co-transfected with both pCMV-agnoprotein (which expresses agnoprotein) and pCMV-GFP (which expresses

25 green fluorescent protein, or "GFP"). In parallel, control U-87MG cells were transfected with pCMV-GFP alone. At 23, 28, 47 and 53 hrs. post-stimulation, cells were harvested and the percentages of cells in G1, S, and G2/M were determined by flow cytometry as described above. The time points measured encompassed one complete round of progression through the cell cycle. Cells

30 that expressed GFP were "gated" on the FACS, and the DNA profile of GFP-positive cells from the pCMV-agnoprotein/pCMV-GFP group was compared to

that of the control cells transfected with only pCMV-GFP. The results are presented below in Table 4.

Table 4 - Cell Cycle Progression of U-87MG Cells Expressing GFP or GFP-Agnoprotein

Hrs. Post Serum Induction	Control (% GFP Cells)			Experimental (%GFP+Agno Cells)		
	G1	S	G2/M	G1	S	G2/M
23	67	17	15	27	21	51
28	29	17	53	28	18	53
47	67	13	19	66	14	18
53	32	15	52	62	12	25

For the pCMV-GFP control cell group, nearly 67% of the cells were found at the G1 stage and only 15% accumulated at the G2/M stage at 23 hrs. after serum stimulation. These values had significantly changed by 28 hrs. post-stimulation, as only 29% of the cells were found in the G1 phase and more than 53% of the cells had progressed to the G2/M phase. At 47 hrs. post-serum stimulation, 67% of the cells were again found at the G1 stage, and only a minor fraction of the cells (19%) were detected at G2/M. As time progressed, more cells departed from the G1 phase and accumulated in G2/M.

In contrast to the control cells, the majority of pCMV-agnoprotein/pCMV-GFP cells (51%) were found at the G2/M phase and only 27% of the cells were detected at G1 stage at 23 hrs. post-stimulation. At 28 hours post-stimulation, 53% of the cells remained at the G2/M stage. At 47 hrs. post-stimulation, the majority of cells (66%) were found at the G1 stage with only a small population of the cells (18%) detected at G2/M. The population of the cells shifted slightly from G1 to G2/M at 53 h post-stimulation, as 62% and 25% of the cells were detected at G1 and G2/M, respectively.

Without wishing to be bound by any theory, from these data it appears that agnoprotein expression prolongs the progression of cells throughout the cell

cycle by stalling cells at the G2/M and G1 stages. This is supported by the observation that, at the earlier hours after serum stimulation (*i.e.*, 4, 12 and 20 hrs.), the population of control and agnoprotein-producing cells which were found at each stage of the cell cycle was virtually identical to those seen at 23 h

5   post-induction.

Example 2 - Suppression of Cell Proliferation and Deregulation of the Cell Cycle by Agnoprotein

Stable NIH 3T3 cell lines that constitutively express agnoprotein were

10   produced using pYFP-agnoprotein, which is a plasmid containing sequences encoding the agnoprotein fused with the mutant variant of GFP called "yellow fluorescent protein" or "YFP". Examination of YFP-agnoprotein production in the stably transfected NIH 3T3 cell lines showed expression of a fusion protein which appropriately localized in the cytoplasmic perinuclear membrane of the

15   cells. Control cells transfected with pEYFP-N1 (expressing YFP alone) showed no evidence for cellular compartmentalization of YFP.

The growth rate of NIH 3T3 cell lines stably expressing either YFP-agnoprotein or YFP alone was examined as follows. An equal number of either cell type was seeded at 20,000 cells/35 mm plate in DMEM plus 10% fetal calf

20   serum. Four independent samples were tested after 24, 48, and 72 hours for cell proliferation by direct counting of the total number of cells, and the experiments were repeated in various stable cell lines to ensure the reproducibility in various clones. Fig. 2A shows an approximately 50% decrease in the number of agnoprotein-producing cells compared to that seen in the control cells.

25   Results from FACS analysis of YFP-producing (control) NIH 3T3 cells revealed that these cells progress abnormally through the cell cycle. At 0 hrs. post serum stimulation, greater than 90% of the YFP-producing cells were found at the G1 phase (Figure 2B). As time progressed, the cells began to enter the S phase and accumulated at G2/M. By 21 hours post-serum stimulation, only 39%

30   of the cells remained at the G1 and greater than 49% of the cells were found at G2/M. At 25 hrs. post-induction, the cells again appeared to depart from G2/M

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and accumulate at the G1 phase. At 29 hrs. post-stimulation, nearly 61% of cells were found at the G1 and only 23% were detected at G2/M.

As shown in Figure 2C, the agnoprotein-producing NIH 3T3 cells exhibited a different pattern of progression through the cell cycle than the
5   control cells. More agnoprotein-producing cells were detected at the G2/M (39.2%) than the control (6.3%) at 0 hrs. post- serum stimulation, and 50% of the agnoprotein-producing cells were found at the G1 stage at this time point. The number of cells at the G1 stage was modestly decreased at 16 and 21 hrs. post-stimulation, and a substantial level of cells were detected at the G2/M
10   phase. Further, a higher number of agnoprotein-producing cells was found at the S phase as compared to control cells. At the later times post-stimulation (*i.e.*, 25 and 29 hrs.), the number of agnoprotein-producing cells at G2/M remained at high levels.

Without wishing to be bound by any theory, these data indicate that
15   expression of agnoprotein deregulates cell cycle progression at various stages, most noticeably by affecting cell departure from G1 to S phase, from S to G2/M, and by inducing an unusual accumulation of cells at G2/M.

Example 3 - Expression of Cyclins A and B and their Kinase Activity
20   ### in Cells Producing Agnoprotein

The level of expression and activity of proteins involved in the control of the cell cycle was examined in NIH 3T3 cells expressing agnoprotein. The levels of cyclin D, cdk4, cdk6, cyclin E and cdk2 levels were comparable in synchronized NIH 3T3 cells expressing YFP (control) and NIH 3T3 cells
25   expressing YFP-agnoprotein prepared as above. Since cyclin A/cdc2 and cyclin B/cdc2 provide the major kinase activity during G2/M, the stage which appears to be affected by agnoprotein, the effect of agnoprotein on cyclin A and cyclin B expression was also examined.

Cyclin A - Cyclin A production was evaluated by Western blot analysis
30   of protein extracts from synchronized NIH 3T3 cells expressing YFP (control) or YFP-agnoprotein prepared as above, at 0, 4, 16, 21 and 29 hours after serum stimulation. Rabbit anti-cyclin A (C-19; Santa Cruz Biotechnology, Inc., Santa

Cruz, CA) was used to probe the blot. Figs. 3A and 3B show a comparable level of cyclin A was expressed in the control and agnoprotein-producing cells. The level of the "housekeeping" protein Grb2 was determined and used a s control for equal protein loading in the gel (see lower panels of Figs. 3A and 3B).

The associated kinase activity of the cyclin A immunocomplex was evaluated by H1 kinase assay. Fig. 3C shows a 35% and 55% decrease in cyclin A-associated kinase activity from agnoprotein-producing protein extracts vs. control cell protein extracts at 16 and 21 hours after serum induction, respectively. Cyclin A-associated kinase activity declined at 29 hrs. after serum induction in the control cells, but not in the agnoprotein producing cells (Fig. 3C).

Cyclin B- Cyclin B levels in synchronized NIH 3T3 cells expressing YFP (control) or YFP-agnoprotein were examined by Western blot analysis at 0, 4, 16, 21 and 29 hrs. after serum stimulation. Mouse anti-cyclin B1 (clone GNS1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to probe the blot. Both control and agnoprotein-producing cells showed a noticeable increase in the level of cyclin B at 21 hrs. post-stimulation in (Figs. 4A and 4B). However, the level of cyclin B in agnoprotein-producing cells was slightly decreased overall vs. control, particularly at 21 hrs. post-stimulation (see Figs. 4A and 4B, lane 4). This difference was not a general event, as the levels of other cellular proteins, including cyclin A (see Figure 3) and "housekeeping" proteins such as Grb2 (see lower panels of Figs. 4A and 4B), remained unchanged in agnoprotein-producing cells relative to control cells.

The associated kinase activity of the cyclin B immunocomplex was evaluated by H1 kinase assay. Fig. 4C shows showed a decrease in cyclin B-associated kinase activity in agnoprotein-producing cells vs. control cells from 16 hrs. post-serum stimulation, including a 40% reduction in kinase activity at 21 hrs. post-serum stimulation.

Example 4 - Expression of Tumor Suppressor Proteins in NIH 3T3 Cells Expressing Agnoprotein

The levels of cell cycle inhibitor proteins p27, p21 and p53 were examined by Western blot analysis on protein extracts from NIH 3T3 cells expressing YFP (control) or YFP-agnoprotein. The blots were probed with anti-p27 and anti-β-actin (Santa Cruz Biotechnology, Inc.); rabbit anti-p21/WAF-1 (Ab-5) and mouse anti-p53 (Ab-1) (Oncogene Science, Boston, MA).

Figs. 5A showed no significant differences in the levels of p27 in the control and agnoprotein-expressing cells. However, the level of p21 was increased in agnoprotein-producing cells as compared to control (Figure 5B). Again, the level of expression of other cellular proteins such as β-actin remained unchanged in the control and agnoprotein-producing cells (Figure 5C).

The ability of agnoprotein to enhance the transcriptional activity of the p21 promoter was also evaluated. Saos-2 cells (which lack endogenous p53) were transfected with plasmid p21-luciferase. Plasmid p21-luciferase has a p21 promoter linked to a luciferase reporter gene, and activation of the p21 promoter causes an increase in luciferase activity.

Saos-2 cells containing the p21-luciferase plasmid were then transfected with either pCMV-Agnoprotein or a plasmid expressing p53, or both. As shown in Fig. 5D, transcriptional activity of the p21 promoter was increased upon expression of either agnoprotein or p53 in transfected Saos-2 cells containing the p21-luciferase plasmid. Co-expression of both agnoprotein and p53 caused a synergistic elevation of p21 promoter activity. These results indicate that agnoprotein increases the level of p21 promoter-driven transcription, and the cooperativity of agnoprotein with p53 can further elevate expression of p21 in cells.

Activation of p21 in agnoprotein-producing cells, which are deregulated in G1 as well as G2/M, is noteworthy in light of recent reports demonstrating that p21 contributes to the regulation of the G2/M transition (see Niculescu AB, III et al. (1998) *Mol. Cell. Biol.* 18, 629-643). Without wishing to be bound by any theory, the activation of p21 in agnoprotein-producing cells may contribute to the de-regulation of G1 as well as G2/M, which leads to growth arrest.

There were also no significant differences in p53 levels of control vs. agnoprotein-producing cells (Fig. 6A). p53 is an upstream regulator of p21. Without wishing to be bound by any theory, these data suggest that a p53 independent pathway may up-regulate expression of p21 in agnoprotein-producing cells.

Example 5 - Association of Agnoprotein with p53

As p53 activity can be regulated through association with other proteins, the ability of agnoprotein to interact with p53 was examined. Protein extracts from NIH 3T3 cells expressing YFP (control) or YFP-agnoprotein were immunoprecipitated with p53-specific antibody pAb 421. Immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Western blot using a rabbit anti-Agno protein as described in DeValle L et al. (2002), *supra*. As shown in Fig. 6D, a distinct agnoprotein-positive band was observed for the extracts from agnoprotein-producing cells, which was absent from the control cell extracts. These data suggest that p53 and agnoprotein are associated in the protein extract.

To verify the interaction of agnoprotein with p53, *in vitro* synthesized [^{35}S]-labeled p53 was incubated with GST alone or with a GST-agnoprotein fusion protein. After 1 hr. incubation at 4° C, the complexes bound to resin were precipitated and washed with binding buffer as described in Safak M et al. (2001), *supra*. Bound proteins were diluted by boiling in Laemmli buffer, resolved by SDS-PAGE, and detected by autoradiography. As shown in Fig. 6B, the 53 kDa [^{35}S]-labeled p53 was retained by the GST-agnoprotein, but not by GST alone.

In the reciprocal experiment, *in vitro* synthesized, [^{35}S]-labeled agnoprotein was incubated with GST or GST-p53, and the bound proteins were resolved by SDS-PAGE followed by autoradiography. As shown in Figure 6C, a band corresponding to agnoprotein was detected in GST-p53 lane, but not in the GST lane.

In a further approach to demonstrate the interaction of agnoprotein and p53, protein extracts from YFP-producing NIH-3T3 cells and control NIH-3T3

cells expressing only YFP were mixed with GST-p53 or GST alone, and the bound proteins were analyzed by Western blot using anti-agnoprotein antibody. As shown in Fig. 6E, a band corresponding the YFP-agnoprotein was observed in GST-p53, but not on GST alone fractions.

To determine the regions of agnoprotein which associates with p53, a GST pull-down assay using fusion proteins in which various regions of agnoprotein were fused to GST was conducted. The agnoprotein fusion protein used in the pull down assay were (with numbers indicating the number of amino acids in the N- to C- terminal direction on the JCV agnoprotein): GST-Agno 1-71; GST-Agno 1-54; GST-Agno 1-36; GST-Agno 36-71; GST-Agno 55-71; GST-Agno 18-54; and GST-Agno 18-71.

As shown in Fig. 6F, the N-terminal region of agnoprotein from amino acids 1 to 36 is sufficient for binding to p53. This region of agnoprotein encompasses a helix-loop-helix structure which has 63% homology with the SV40 agnoprotein.

Thus, it is evident that agnoprotein is able to interact with p53. Without wishing to be bound by any theory, the interaction of agnoprotein and p53 may affect other events involved in the control of cell cycle progression (such as expression of downstream targets of p53) and contribute to the inhibition of cell growth effected by agnoprotein.

All documents referred to herein are incorporated by reference. While the present invention has been described in connection with the preferred embodiments and the various figures, it is to be understood that other similar embodiments may be used or modifications and additions made to the described embodiments for performing the same function of the present invention without deviating therefrom. Therefore, the present invention should not be limited to any single embodiment, but rather should be construed in breadth and scope in accordance with the recitation of the appended claims.